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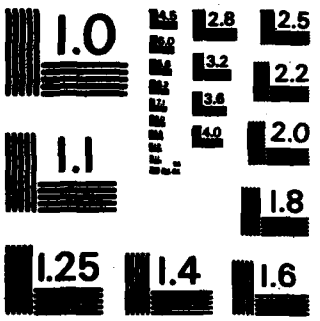

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Initially, the objectives of this work were to characterize substances which regulated DNA synthesis in lymphocytes. In particular we wished to determine the role of detergent soluble(DS)DNA in replication of activated splenocytes. We now feel that DS DNA is a hitherto overlooked class of replication intermediates not yet stabilized by nucleosome maturation. One possibility is that the detergent lability arises because of the presence of topoisomerase II in the replicating region which results in the release of these fragile fragments. These observations hold not only in activated lymphocytes but also in any actively dividing cell.

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FINAL REPORT

OFFICE OF NAVAL RESEARCH - IMMUNOLOGY PROGRAM

N00014-82-K-0283, LYMPHOCYTE ACTIVATION-REGULATORY SUBSTANCES

PHYLLIS R. STRAUSS, PRINCIPAL INVESTIGATOR

1.0 Experimental Objectives

Initially the objectives of this work were to characterize substances which regulated DNA synthesis in lymphocytes. In particular, we wished to determine the role of detergent soluble (DS)DNA in replication of activated splenocytes. DS DNA is that fraction of DNA released from chromatin by non-ionic detergent treatment of dividing cells and it labels more efficiently with ^{3}H-thymidine than bulk chromatin which remains in the detergent insoluble (DI) fraction.

2.0 Detailed final report

While DS DNA has been observed in the past by a number of investigators, it has been dismissed by others as an artifact. We now feel that rather than being an artifact, DS DNA is a hitherto overlooked class of replication intermediates not yet stabilized by nucleosome maturation. One possibility is that the detergent lability arises because of the presence of topoisomerase II in the replicating region which results in the release of these fragile fragments. These findings hold not only in activated lymphocytes but also in any actively dividing eukaryotic cell. Lymphocytes may differ in the amount of DNA available to detergent solubilization at any time.

The evidence for this hypothesis is based on both unpublished and previously published data, which is described below.

2.1 DNA solubilized from dividing cells is heterogeneous in nature and not mitochondrial DNA. DNA obtained from the DS fraction of dividing splenocytes represents 1-3% of total cellular DNA. The mild detergent treatment of whole cells (0.1%-0.5% Nonidet P40) is performed rapidly at 4 C by microfuge centrifugation in the presence of 25mM EDTA and protease inhibitors such as PMSF, bisulfite and antipain. EDTA and the protease inhibitors are not necessary to obtain DS DNA. Note that topoisomerase II is active in the presence of EDTA and that detergent (often SDS) is usually used to stabilize the DNA fragments in vitro so they are not re-ligated.

While mitochondrial DNA can be isolated from the DS fraction, there is so little that detection usually requires Southern blot hybridization. Furthermore, the two mitochondrial DNA bands have different mobilities than the bulk of DS DNA. Cot analysis of DS DNA using whole mouse genomic DNA as the driver reveals sequences with slow, medium and rapid annealing kinetics. Furthermore, every genomic probe we have used shows homology by Southern blot hybridization. In short the DNA in the DS fraction is heterogeneous in informational content as would be expected of replication intermediates.

2.2 DNA obtained in the DS fraction labels efficiently with ^{3}H-thymidine and to a higher specific activity than DNA from the detergent insoluble fraction. It labels with all four nucleoside precursors as expected for replication intermediates. However, exposure to ^{3}H-thymidine or other radionuclides is not necessary to generate DS DNA, and we often prepare DS DNA in bulk from unlabeled cells. Therefore, DS DNA is not generated under our conditions through the action of radionuclide decay. Also, we have taken care to determine which cell lines are sensitive to either ^{3}H-thymidine or unlabeled exogenous pyrimidine by viability assays and growth curves and to work within the constraints of each system. The shortest pulse that we have used is 5 minutes. Experiments using shorter pulses are in progress. What has kept us from performing these experiments as a first priority is the requirements for very high concentrations of cells (possibly 10^8 cells/ml instead of 5×10^6/ml) and very high concentrations of radiolabel (50-100 μCi/ml instead of 1-6 μCi/ml). Many replication studies employ high cell and/or radiolabel concentrations without regard for effects on viability and growth potential and the results might be explained as the result of radionuclide decay.

2.3 Incorporation of ^{3}H-thymidine into DS DNA is inhibited by aphidicolin and hydroxyurea, inhibitors of polymerase$_\alpha$ and ribonucleotide reductase respectively. The amount of DNA recovered in the DS fraction remains unchanged. Inhibition of ^{3}H-thymidine incorporation without alteration of amount is expected if the DNA in the DS fraction contains replication intermediates unable to proceed in the replication process.

2.4 Inhibitors of topoisomerase II inhibit DS DNA. Novobiocin, which inhibits the topoisomerase associated ATPase activity, stops incorporation of ^{3}H-thymidine into DNA in the DS fraction without altering the amount of recovered DNA. The effect is reversible. On the other hand, mAMSA, which forms a ternary complex with topoisomerase II and DNA, alters both the amount of recovered DNA and the labeling kinetics. At low concentrations of mAMSA, (2-5 μM) incorporation of ^{3}H-thymidine is inhibited while the amount of DS DNA is enhanced. At higher concentrations not only is ^{3}H-thymidine incorporation stopped but also the amount of DNA recovered in the DS fraction is markedly diminished. The mAMSA studies are reminiscent of results reported by Scheinin in which the tsAIS9 cell line, temperature sensitive for topoisomerase II, accumulates low molecular weight DNA at the non permissive temperature. VM26, which binds to the enyzme and prevents its insertion into double stranded DNA, also inhibits ^{3}H-thymidine incorporation at low concentrations and reduces the amount of DNA released into the DS fraction. Because these inhibitors alter both labeling kinetics and amounts of DNA recovered, an artifact caused by detergent (such as peroxidation) becomes unlikely. Futhermore, in preliminary studies where we examined DNA from the DS fraction by electron microscopy the molecules appear to be double stranded, an observation that is consistent with the mode of action of topoisomerase II which makes and seals double stranded cuts. Our concern is to exclude the possibility that endogenous nucleases unrelated to replication (so called "promiscuous" nucleases) are the cause of lability during the detergent treatment. Since inhibitors of topoisomerase II result in depletion of DS DNA, "promiscuous" nucleases are no longer an issue.

2.5 DS DNA is present in a wide variety of cell types. Among the cell types from which DS DNA can be recovered are splenocytes from immunologically activated mice, erythroblasts from phenylhydrazine treated anemic mice, various mouse and human lymphocyte lines (T and B), murine erythroleukemia lines, Chinese hamster ovary cells, HeLa cells and finally Drosophila melanogaster and Xenopus laevis embryos but not inactive Xenopus oocytes. The last source is particularly important because Xenopus embryos and activated oocytes (activated by microinjection) contain very little "promiscuous" nuclease activity not associated with the replication apparatus. Xenopus embryos contain so little nuclease activity that both single stranded and double stranded exogenous DNA can be microinjected and recovered intact, indeed, replicated. Therefore, if dividing Xenopus embryos makes DNA which can be extracted into the DS fraction and if the DNA is not an artifact of detergent solubilization, the DNA in the DS fraction is likely to represent replication intermediates.

2.6 Cycloheximide, which inhibits both protein synthesis and DNA chain elongation, inhibits incorporation of ^{3}H-thymidine into DNA in the DS fraction. No change in amount of DS DNA is observed. This observation may imply that synthesis of proteins important for replication and chromatin maturation (possibly histones but see below) have ceased but it cannot be excluded that the primary event is cessation of DNA chain elongation under these conditions.

2.7 Chromatin maturation is not required for the generation of DS DNA. Sodium butyrate inhibits histone deacetylation required for chromatin maturation, increases the amount of DNA recovered in the DS fraction and the incorporation of ^{3}H-thymidine. Treatment of splenocytes from concanavalin A- stimulated mice with sodium butyrate is without effect. The results are observed under conditions where the inhibition of histone deacetylation is confirmed. Therefore, it would appear that chromatin maturation as affected by histone deacetylation is not required for the generation of DS DNA. If anything, inhibiting chromatin maturation increases the availability of DNA to be released into the DS fraction.

2.8 DS DNA is not nucleosomal. When purified DS DNA is resolved by electrophoresis employing 1% or 1.2% agarose under native conditions, DS DNA is seen to consist of a ladder of size classes reminiscent of nucleosomal ladders generated by nucleases exogenously added to isolated nuclei. The possibility that DNA is detergent soluble because endogenous, "promiscuous" nucleases had cleaved intact chromatin was initially so persuasive that we decided to perform a simple experiment to compare the "nucleosomal equivalents" (moles of double stranded DNA equivalent to unit lengths) in the DS fraction with moles histone octamers from the same preparation. The comparison revealed that there is about 5% the amount of histone necessary to account for DNA in the DS fraction if it were released nucleosomes. If ^{3}H-labeled histones isolated from the detergent insoluble fraction are mixed with cold detergent supernate and the mixture re-extracted for histones 65-100% of the starting ^{3}H-histones are recovered. Furthermore, while slight loss of H3 and H4 are observed, the bulk of the recovered ^{3}H-histones material behaves as the control on SDS polyacrylamide gel electrophoresis followed by autoradiography. As a test of our ability to isolate histones and therefore to make these quantitative estimates, we determined that the molar ratio of histone to "nucleosome equivalents" for chromatin remaining

in the detergent insoluble fraction was 0.89 and 1.0 in two separate experiments. Hence, it would appear that the DS DNA is not associated with histone octamers.

Upon sucrose gradient sedimentation (18hrs at 28,000 rpm, 5%-30% gradient with a 2M sucrose cushion) DNA in the crude detergent lysate behaves in a particulate fashion with an array of size classes. If the gradient contains 2M NaCl, the sedimentation pattern collapses into a single band just below the top of the gradient and all size classes are recovered from that band. Unlike nucleosomes generated from calf thymus by micrococcal nuclease, whatever histones are present in the detergent soluble fraction do not co-sediment with the DNA. We are currently analyzing our most recent results to determine whether there are characteristic protein composition for the particulate structures from the detergent soluble DNA.

Finally, the DNA in the crude detergent soluble fraction is protected from micrococcal nuclease as would be expected of a nucleoprotein structure. In short DNA from the DS fraction behaves as if it were replication intermediates in particulate structures generated through the involvement of topoisomerase II. Is it possible that eukaryotic cell DNA replication contains a step which has been overlooked in the past which involves topoisomerase II?

2.9 DNA which is not packaged into nucleosomes still can form DS DNA. In collaboration with Dr. John Bodnar we have recently shown that DNA with the same characteristics to those described above can be isolated from HeLa cells infected with adenovirus-2 late in infection when only the virus is replicating. Virologists prefer to isolate adenovirus from nuclei obtained through detergent lysis and it would appear that they routinely discard these forms. There is general agreement that adenovirus DNA does not utilize host histones and does not mature into nucleosomes. Rather adenovirus develops its own nucleoprotein particle using protein VII, a virally encoded product. These observations strengthen our conviction that DS DNA is a nucleoprotein structure distinct from nucleosomes.

3.0 Publications/presentations related to this project

3.1 Publications

3.1.1 STrauss, P.R., LaGree, K.A. and Mui, S.C. 1983 Pulse labeling of metabolically active detergent soluble DNA. J. Cell Biol. 97: 113a.

3.1.2 Strauss, P.R., Andrutis, A.T., Leong, S., Nickeson, S. and Supple, E. 1984 Metabolically active detergent soluble DNA in murine splenocytes. Biochemistry (Wash.) 23: 915-921.

3.1.3 Strauss, P.R., Banerjee, P.T., LaGree, K.A. and Mui, S.C. 1984 Kinetics of thymidine incorporation into detergent soluble (DS)DNA of mouse lymphocytes. Proc. Natl. Acad. Sci. (USA) 81: 7056-7060.

3.1.4 Andrutis, A.T. and Strauss, P.R. 1984 Cell cycle dependence of ^{3}H-thymidine incorporation into metabolically active detergent soluble DNA from a human T cell (Jurkat) line. Fed. Proc. 43: 1996.

3.1.5 Stetten, G., Andrutis, A.T. and Strauss, P.R. 1984. The human Jurkat T cell line is hypotetraploid. In: International Cell Biology 1984 Ed. S. Seno and Y. Okada Academic Press Japan, Inc. Tokyo

3.1.6 Strauss, P.R., LaGree, K.A. and Mui, S.C. 1984 Is detergent soluble DNA related to nucleosomes? J. Cell Biol. 99: 9a.

3.1.7 Strauss, P.R., 1985 Incorporation of [^{3}H]nucleosides and [^{3}H]deoxynucleosides into detergent soluble DNA. Proc. Soc. Exp. Biol. Med. 179: 487-491.

3.1.8 Strauss, P.R., Henderson, J.F. and Goodman, M.G. 1985 Nucleosides and lymphocytes - an overview. Proc. Soc. Exp. Biol. Med. 179: 413-418.

3.1.9 Zhang, L.H., Mui, S.C., Todt, J.T. and Strauss, P.R. 1985 Topoisomerase II is required for synthesis and turnover of detergent soluble (DS)DNA J. Cell Biol. 101: 460a.

3.1.10 Strauss, P.R. 1985 Lymphocytes and lymphocyte lines secrete adenosine deaminase. In: Purine Metabolism in Man-V, Nyhan, W.L. Ed. Plenum Press, New York. In press.

3.1.11 Andrutis, A.T., LaGree, K.A., Stetten, G. and Strauss, P.R. 1985. Detergent soluble (DS)DNA in the human Jurkat cell line. Cancer Res. Manuscript submitted.

3.1.12 Strauss, P.R., LaGree, K.A., Mui, S.C. and Todt, J.T. 1985 Detergent soluble DNA is not associated with core histones. Nucleic Acids Res. Manuscript under revision.

3.1.13 Zhang, L.H., Mui, S.C., Todt, J.T. and Strauss, P.R. 1985. The role of topoisomerase II in synthesis and turnover of detergent soluble (DS)DNA. Proc. Natl. Acad. Sci. (USA) Manuscript submitted.

3.1.14 Mui, S.C. and Strauss, P.R. 1985. Cyclohexamide but not sodium butyrate interferes with incorporation of [^{3}H]thymidine into detergent soluble (DS)DNA. Manuscript in preparation.

3.2 Presentations

3.2.1 Invited lectures

1982 University of Glasgow, Glasgow Scotland
Department of Biology, Northeastern University
1984 ONR Immunological Defense Investigators Meeting
Department of Pharmacology, Kawasaki Medical School, Kurashiki, Japan
Department of Biophysics, Kyoto University, Kyoto, Japan
Chromatin Workshop, Tokyo, Japan
1985 Department of Biology, The Johns Hopkins University
Department of Radiobiology, University of California at San Francisco

3.2.2 Papers presented

1982 American Society of Cell Biology, Baltimore
1983 American Society of Cell Biology, San Antonio
1984 Third International Congress of Cell Biology, Tokyo
American Society of Cell Biology, Kansas City
Conference on Nucleosides and Lymphocytes, Weston, MA (co-organizer)
1985 Fifth International Symposium on Human Purine and Pyrimidine Metabolism, LaJolla, CA

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